

Cooperative Action between Band 3 and Glycophorin A in Human Erythrocytes: Immobilization of Band 3 Induced by Antibodies to Glycophorin A

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ABSTRACT The ability of transmembrane receptor proteins to change their association with the cytoskeleton in response to ligand binding seems to be a key mechanism of signal transduction across membranes. To investigate the molecular features of this mechanism we have used the red cell membrane as a model system to study signal transduction through the integral protein, glycophorin A. In these studies the lateral mobility of integral proteins was measured in situ by fluorescence recovery after photobleaching, and membrane rigidity was characterized by micropipette aspiration technique. We found that binding either a monoclonal antibody or its monovalent Fab to the exoplasmic domain of glycophorin A in normal red cells immobilized the receptor and rigidified the membrane. Further, immobilization and rigidification did not occur when antibodies were bound to Miltenberger V cells containing a mutant form of glycophorin A lacking the cytoplasmic domain. These results imply that the site of the immobilization/rigidification lies within the membrane skeletal structure, not in exofacial receptor crosslinking, and requires the extended cytoplasmic domain of normal glycophorin A. In addition, we found that glycophorin A immobilization and membrane skeletal rigidification were accompanied by immobilization of band 3 receptors. This unexpected result indicates a cooperative coupling between liganded glycophorin A, band 3, and the membrane skeleton. We speculate that cooperation of this type may represent a general mechanism for cytoskeletal linkage and transformation initiated by receptors with short cytoplasmic sequences, such as integrins.

INTRODUCTION

Receptor-mediated communication between extracellular environments and the cytoplasmic chemistry of cells is a universal sensory mechanism in biology. As a subclass of this phenomena, extracellular binding of specific macromolecules to eukaryotic cell receptors initiates transmembrane signals to the cytoskeletal structure, which plays a central role in, for example, tissue development, neuromuscular action, and immune defense. Using the red blood cell membrane as a model system, we have begun to study the mechanism of signaling involved in ligand-induced changes in the membrane skeleton.

Well characterized biochemically, the red blood cell membrane is composed of a lipid bilayer, integral proteins, and a spectrin-based skeletal protein network associated with the bilayer via protein-protein and protein-lipid interactions (Bennett, 1985; Chasis and Mohandas, 1992; Liu and Derick, 1992; Gilligan and Bennet, 1993; Tanner, 1993). Ample evidence supports an important role for the skeletal protein network in determining biophysical properties of the membrane (Evans and Hochmuth, 1977; Fischer et al., 1978; Liu and Palek, 1980; Smith and Palek, 1983; Mohandas and Chasis, 1993; Mohandas and Evans, 1994). In earlier studies we demonstrated that binding of ligands (either monoclonal an-

tibodies or their monovalent Fab fragments) to the integral protein glycophorin A produced a marked increase in membrane rigidity as measured by ektacytometry (Chasis et al., 1985). The cytoplasmic domain played a crucial role in this process, inasmuch as ligand binding did not induce rigidity in Miltenberger V (MiV) red cells in which a variant of glycophorin A lacks most of its cytoplasmic domain (Chasis et al., 1988). Because the skeletal protein network regulates membrane deformability, these results suggested that ligand binding can initiate a transmembrane signal that causes an increased interaction of the cytoplasmic domain of the receptor with the underlying cytoskeletal network.

To test this hypothesis and to investigate the molecular features of such a mechanism, the effect of ligand binding on the lateral mobility of three integral proteins, glycophorin A, glycophorin C, and band 3, was measured in both normal and variant red blood cells. Lateral mobility of the integral proteins was measured in situ by the technique of fluorescence recovery after photobleaching (FRAP). This technique involves 1) quantitating the fluorescence intensity from labels conjugated to the ligand or receptor of interest on the membrane, 2) irreversibly bleaching a small region of the membrane, and 3) measuring the subsequent fluorescence recovery due to lateral motion of fluorophore on the surface. In addition, micropipette aspiration of ligand-bound discocytes was used to expose rigidification of the membrane skeleton; rigidity was quantitated by the increase in suction versus length inside the pipette.

We found that binding either a monoclonal antibody or its monovalent Fab to glycophorin A in normal intact red cells both immobilized the receptor and rigidified the membrane.

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Further, immobilization and rigidification did not occur when antibodies were bound to MiV cells. These results imply that the site of the immobilization/rigidification lies within the membrane skeletal structure, not in exofacial receptor crosslinking, and requires the extended cytoplasmic domain of normal glycophorin A. In addition, we found that glycophorin A immobilization and membrane skeletal rigidification were accompanied by immobilization of band 3 receptors. This unexpected result indicates a cooperative coupling between liganded glycophorin A, band 3, and the membrane skeleton. We speculate that cooperation of this type may represent a general mechanism for cytoskeletal linkage and transformation initiated by receptors with short cytoplasmic sequences, such as integrins.

MATERIALS AND METHODS

Cells

After obtaining informed consent, blood from normal volunteers and from an individual with MiV blood type was collected in acid-citrate-dextrose. Llama blood was generously provided by Dr. Joe Smith (Kansas State University, Manhattan, KS). Whole blood was washed and diluted for experiments in phosphate-buffered saline solution (290 mOsm, pH 7.4) containing 0.05g% human serum albumin (PBS/HSA). MiV and normal "travel control" blood samples were stored at 77°K and fast thawed when needed.

Receptor Labeling

Affinity-purified monoclonal antibodies to glycophorin A (R10) and glycophorin C (Bric 10) were generously supplied by Dr. David Anstee (South Western Regional Blood Transfusion Center, Bristol, UK). Monovalent Fab fragments were prepared as previously described (Mischell, 1980), and their monovalency was checked by electrophoresis of reduced and unreduced samples in the discontinuous system of Laemmli (1970). Antibodies and Fab fragments were fluorescently labeled after dialysis in 0.2 M sodium borate, pH 9.4. FITC was dissolved in DMSO (1 mg/ml) and added to protein solutions at 30 μ l of FITC/mg of protein. The samples were stirred for 2 h at room temperature and fluoresceinated protein purified by gel filtration over sephadex G-25 (Pharmacia, Alameda, CA) followed by extensive dialysis in PBS, pH 7.5.

Band 3 was labeled in situ with eosin-5-maleimide (EMA) (Molecular Probes, Eugene, OR) using techniques modified from Nigg and Cherry (1979) and Golan (Golan et al., 1986). 10 μ l of whole blood was suspended and washed 3 times in 1 ml of PBS/HSA at 4°C. The cells were then incubated at room temperature for 30 min in 50 μ g/ml of EMA dissolved in PBS/HSA and then washed 3 times in PBS/HSA buffer.

The extracellular domain of glycophorin A was liganded with R10 or R10-Fab by incubating 0.3 μ l of packed red cells in a solution of the antibody at the required concentration for 30 min at room temperature.

Lateral mobility measurements

The lateral motion of fluorescently labeled cell surface receptors was determined by FRAP (Jacobson et al., 1976; McGregor et al., 1984; reviewed by Wolf, 1989). Briefly, an argon ion laser beam (476 nm at 350 mW) was optically split and recombined to produce 2 coaxial beams with the ratio of intensities of 100–1000:1. Each beam was shutter controlled by computer and focused by an epiilluminated fluorescence microscope to produce a diffraction-limited spot with Gaussian intensity profile (waist diameter $0.55 \pm 0.05 \mu$ m) at the object plane of the microscope. The less intense beam stimulated pre- and postbleach fluorescence emission from the desired area on the cell surface. The fluorescence intensity was quantified by a photomultiplier tube. The area was first irreversibly photobleached by a short

pulse by the intense beam. The subsequent fluorescence recovery, due to lateral redistribution of the fluorophor, was then quantified using the less intense beam with the photomultiplier. Fluorescence recovery curves were fit with the linearized solution of Yguerabide (Yguerabide et al., 1982) to yield least square fit values for the diffusivity ($D \text{ cm}^2/\text{sec}$) and the recovery percentage ($R\%$) of the receptor. All experiments were performed at room temperature ($24 \pm 1^\circ\text{C}$).

Membrane Rigidity Measurements

The extensional rigidity of the red cell membrane was evaluated by micropipette aspiration technique as previously described (Evans et al., 1984). In brief, membrane extensional rigidity (μ) was derived from observation of cell length (L) aspirated into a small micropipette (with radius R_p) in response to the increase in suction pressure (P). Analysis of this experiment has shown that μ is proportional to the derivative of the pressure with respect to length: $\mu \sim R_p^2 \times dP/dL$.

RESULTS

Antiglycophorin A immobilizes its receptor and rigidifies the membrane in normal red cells

To study the effect of ligand binding on the lateral mobility of glycophorin A, the monoclonal antibody R10 was used. R10 binds to the exoplasmic domain of glycophorin A in the region of amino acid residue 35 and does not bind to erythrocytes pretreated with trypsin (Anstee and Edwards, 1982; Bigbee et al., 1983). Initially, binding isotherms were performed to determine the antibody concentrations at which saturation occurred. Subsequent experiments were then performed using saturating concentrations.

When normal erythrocytes were liganded with FITC-labeled R10 and R10 Fab at saturation the mobile fraction of glycophorin A was less than 10% (Fig. 1). Membrane rigidification (demonstrated originally by ektacytometry; Chasis et al., 1985) was confirmed by the single cell micropipette assays. Normal cells with increasing amounts of bound R10 and R10 Fab showed increasing rigidity in a dose-dependent manner. These observations indicate that membrane rigidity induced by R10 binding is associated with a completely immobile population of glycophorin A, and that neither rigidification nor immobilization result from extracellular crosslinking, inasmuch as monovalent Fab and bivalent IgG produced similar results.

Glycophorin A receptors are not immobilized by antiglycophorin A in MiV red cells and cell deformability remains unchanged

To study the role of the cytoplasmic domain of glycophorin A in ligand-induced rigidification and receptor immobilization we used homozygous MiV red cells. In individuals homozygous for this phenotype, the membrane contains no normal glycophorin A or glycophorin B but instead contains a hybrid sialoglycoprotein composed of the extracellular domain of glycophorin A and the intramembranous and cytoplasmic domains of glycophorin B (Vignal et al., 1990; Huang and Blumenfeld, 1991). The binding isotherm of R10 to MiV cells was similar to that observed in normal red cells,

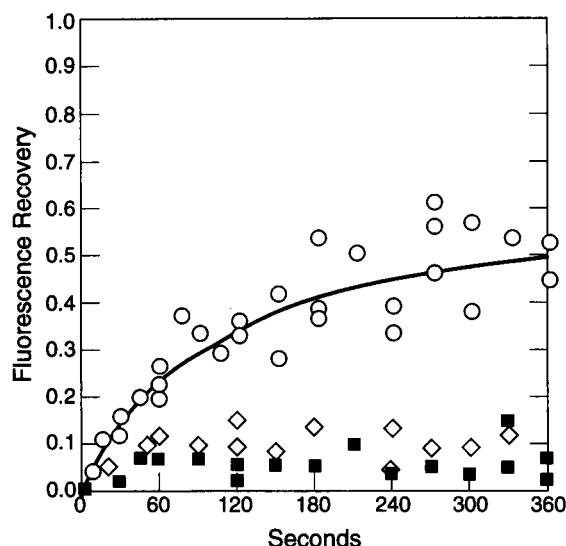


FIGURE 1 Fluorescence recovery of liganded glycophorin A in normal and MiV erythrocytes. When normal red cells were liganded with FITC-labeled R10 (■) and R10 Fab (◇) at saturation, the mobile fraction of glycophorin A was less than 10%. In contrast, when MiV cells were liganded with FITC-labeled R10 (○), the hybrid glycophorin had a mobile fraction of 69%. (■ data points are measurements from 8 cells; ◇ data points are measurements from 6 cells; and ○ data points are measurements from 10 cells).

except that the fluorescence yield at saturation was reduced by 50%. This finding supports earlier reports that the copies/cell of variant glycophorin in MiV cells is half that of glycophorin A in normal cells (Merry et al., 1986). The hybrid glycophorin bound at saturation with FITC-labeled R10 had a mobile fraction of $69 \pm 3\%$ (Fig. 1). Further, the elastic shear modulus measured by micropipette assay was markedly increased in normal erythrocytes after ligand binding (Table 1). No such increase in rigidity was noted after ligand binding to MiV erythrocytes (Table 1). These observations strongly support the thesis that R10-induced rigidification and immobilization is caused by an interaction involving the cytoplasmic domain of glycophorin A. The data also clearly indicate that extracellular cross-linking plays no role in this process.

Band 3 receptors are immobilized by antiglycophorin A in normal red cells but not in MiV red cells

Because band 3 has a long cytoplasmic domain that binds to ankyrin in the skeletal network and there is evidence of close

TABLE 1 Effect of antiglycophorin A on the membrane rigidity of normal and MiV* red cells

	Rigidity ($\mu\text{N}/\text{m}$)
Normal erythrocytes	
without antibody	8 ± 1
with antibody (100 $\mu\text{g}/\text{ml}$)	117 ± 50
MiV erythrocytes	
without antibody	8 ± 1
with antibody (100 $\mu\text{g}/\text{ml}$)	8 ± 1

* MiV, Miltenberger V.

physical proximity of glycophorin A and band 3 on the red cell membrane, we questioned whether band 3 cytoplasmic domain might contribute to glycophorin A ligand-induced rigidification. To address this issue band 3 molecules in normal cells were labeled with EMA, and then the cells were incubated in varying concentrations of unlabeled R10. As shown in Fig. 2, EMA-labeled band 3 in normal cells had a mobile fraction of $43 \pm 6\%$. The mobile fraction of band 3 decreased in a dose-dependent fashion as the concentration of R10 increased. At saturation, band 3 was completely immobilized. In contrast, EMA-labeled band 3 in MiV cells treated with R10 had normal lateral motion and a mobile fraction of $41 \pm 4\%$ (Fig. 3). These observations imply that the cytoplasmic domain of glycophorin A plays a crucial role in R10-induced band 3 immobilization.

To exclude the possibility that R10 binding nonselectively affected the lateral mobility of transmembrane proteins, we measured its effect on the lateral mobility of another integral protein, glycophorin C. For these studies glycophorin C molecules were fluorescently labeled with FITC BRIC 10, a monoclonal antibody specific for the exoplasmic domain of glycophorin C (Anstee et al., 1984; Dahr et al., 1989). These labeled erythrocytes were then incubated with varying concentrations of unlabeled R10 (0–100 $\mu\text{g}/\text{ml}$). As depicted in Table 2, R10 binding reduced the diffusivity of glycophorin C in a dose-dependent fashion but did not alter its mobile fraction, indicating that glycophorin C was not immobilized, but that its lateral motion simply was hindered. We concluded that R10-induced immobilization of band 3 and glycophorin A was a specific effect on these two integral proteins.

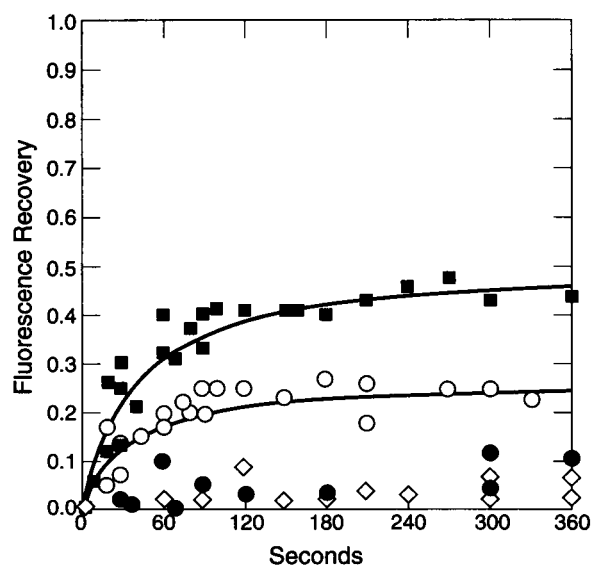


FIGURE 2 Effect of antiglycophorin A antibody on the fluorescence recovery of band 3 in normal erythrocytes. When red cells were labeled with EMA and then incubated in varying concentrations of unlabeled R10, EMA-labeled band 3 had a mobile fraction of $43 \pm 6\%$ (■), EMA-labeled band 3 in cells treated with 2.5 $\mu\text{g}/\text{ml}$ R10 had a mobile fraction of 28% (○); EMA-labeled band 3 in cells treated with 100 $\mu\text{g}/\text{ml}$ R10 had a mobile fraction of less than 10% (●, ◇). (■, ○, and ● data points are measurements from 8 cells; ◇ data points are measurements from 5 cells.)

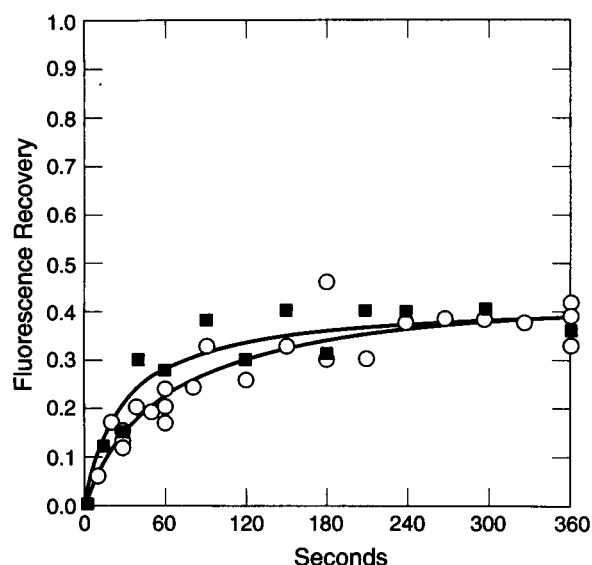


FIGURE 3 Effect of antiglycophorin A antibody on the fluorescence recovery of band 3 in MiV erythrocytes. EMA-labeled band 3 had a mobile fraction of $41 \pm 4\%$ in both control, nonliganded cells (■) and in cells liganded with unlabeled R10 antibody at $100 \mu\text{g/ml}$ (○). (■ data points are measurements from 6 cells and ○ are data points from 8 cells.)

TABLE 2 Effect of glycophorin A ligand binding on the lateral mobility of glycophorin C

R10 Concentration ($\mu\text{g/ml}$)	GPC* Diffusion $D(\text{cm}^2/\text{s})$	GPC Mobile Fraction (%)
0	$(1.6 \pm 0.5) \times 10^{-11}$	43.7 ± 5
1	$(0.61 \pm 0.2) \times 10^{-11}$	40 ± 10
100	$(0.18 \pm 0.09) \times 10^{-11}$	44 ± 15

* GPC: glycophorin C.

Band 3 receptors are immobilized in normal llama red cells

Llama erythrocytes have been shown by ektacytometry to have markedly increased membrane rigidity (Smith et al. 1980). Furthermore, biochemical analysis suggests that the structure of llama band 3 cytoplasmic domain differs from that of normal, human band 3 (Khodadad and Weinstein, 1985). We were, therefore, interested in analyzing the mobile fraction of band 3 in these animal erythrocytes. As shown in Fig. 4, the lateral mobility of EMA-labeled band 3 was markedly decreased. In contrast, ETSC-labeled molecules (which are sialic acid-containing integral proteins, predominantly the various glycophorins, Golan, 1989) had high fractional recovery (60–80%) in llama cells. A value similar to that was also observed for ETSC-labeled molecules in normal, human erythrocytes. These data indicate that immobilization of band 3 is a distinct feature of llama red cells. Similar results have recently been reported for the rigid ovalocytic human red cells in which band 3 but not glycophorin is immobilized (Liu et al. 1990; Mohandas et al. 1992).

DISCUSSION

Our results show that in normal human red cell membranes, the binding of ligands to the extracellular domain of glyco-

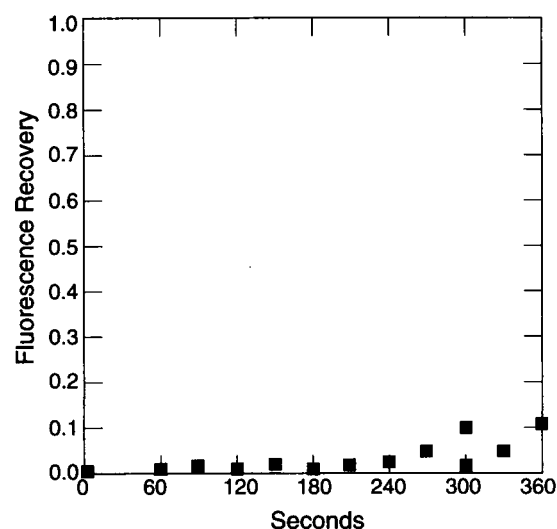


FIGURE 4 Fluorescence recovery of band 3 in llama erythrocytes. The mobile fraction of EMA-labeled band 3 was less than 10% (■ data points are measurements from 11 cells).

phorin A causes marked reductions of the mobile fraction of glycophorin A. At saturation, the binding of fluorescently labeled R10 and its Fab fragment caused the mobile fraction to fall to $10 \pm 3\%$, indicative of near complete glycophorin A immobilization within the temporal window of the FRAP experiment ($\sim 30 \text{ min}$). This value places an upper bound on the diffusivity of liganded glycophorin A of slightly less than $10^{-13} \text{ cm}^2/\text{s}$ if one assumes that the mobile fraction is 60% in the infinite time limit. This diffusivity of liganded glycophorin A value is a reduction of 2 orders of magnitude from the diffusivity of unliganded receptor proteins and is the same as that estimated for the diffusivity of spectrin in the cytoskeleton (Peters et al., 1974), suggesting that ligand-induced immobilization of glycophorin A is due to an interaction of glycophorin A with the cytoskeletal network. If immobilization of glycophorin A is, indeed, occurring through an interaction with the skeletal network, then the cytoplasmic domain of the sialoglycoprotein will play an essential role in this process. To test this thesis, we studied ligand binding to MiV erythrocytes that contain no normal glycophorin A molecules but instead possess a mutant sialoglycoprotein encoded by a gene composed of exons 1 through 3 of the glycophorin A gene and exons 3 through 5 of the glycophorin B gene (Vignal et al., 1990; Huang and Blumenfeld, 1991). The resulting hybrid glycoprotein is composed of the exoplasmic domain of glycophorin A and the transmembrane and cytoplasmic domains of glycophorin B. Hence, this mutant protein contains only 6 amino acids in its cytoplasmic domain compared with the 39 residues present in the normal protein. Because the exoplasmic domain of the MiV variant contains the antigenic epitope for R10, the effect of ligand binding on the lateral motion of the variant protein could be measured. Interestingly the variant glycophorin was highly mobile in these cells after binding of R10 in contrast to the observed low mobile fraction of liganded glycophorin A in normal cells. These observations imply that ligand binding

does not induce immobilization of the hybrid glycoprotein and clearly suggest that the cytoplasmic domain of glycoporphin A plays a key role in immobilization. This conclusion also was supported by measurements of membrane rigidity.

One important issue to address is the mobility of glycoporphin A in the nonliganded state. Previous studies by Golan and others (Golan, 1989), as well as our own, have shown that glycoporphin A labeled with ETSC has a mobile fraction of 60–70% in normal red cells. Although ETSC labels glycoporphin A, it also labels other sialic acid-containing moieties on the cell surface. Therefore, unlike EMA labeling of band 3, ETSC is not a specific label for glycoporphin A. For this reason we believe our data on MiV membranes specifically labeled with antibody to glycoporphin A are a better indication of native glycoporphin A mobility. In addition, we observed a reduction in the mobile fraction of the ETSC-labeled molecules after binding of antibody to glycoporphin A. In light of these data, we are confident that ligand binding induces immobilization of glycoporphin A.

The puzzling question is how does the relatively short 39-residue cytoplasmic domain of glycoporphin A interact with the skeletal proteins to induce rigidification? One clue may be in the unexpected results obtained with the EMA-labeled cells. In these studies progressive immobilization of the endogenously labeled band 3 receptors occurred in a dose-dependent manner with increasing concentrations of R10 antibodies. Indeed, at saturation binding, the band 3 mobility fell to $7 \pm 3\%$, suggesting complete band 3 immobilization. The observed concurrent immobilization of band 3 and glycoporphin A indicates that these two integral proteins may interact cooperatively with the membrane skeleton when glycoporphin A is liganded. Further, it is clear that the interaction requires the cytoplasmic domain of glycoporphin A, inasmuch as band 3 was not immobilized in mutant MiV red cell membranes when R10 was bound at saturation. For cooperative interaction to occur, we expect that band 3 and glycoporphin A molecules should be in close proximity

to one another in the membrane; two lines of evidence suggest that this may be so. First, Nigg and colleagues reported that antiglycoporphin A antibodies markedly reduced the rotational diffusion of band 3 and concluded that band 3 and glycoporphin A might form a complex in the membrane (Nigg et al., 1980). Second, we have previously shown that band 3 and glycoporphin A are both involved in W_r^b antigen expression, which would necessitate close proximity of these two integral proteins in the membrane (Telen and Chasis, 1990). Other evidence also supports the thesis that regulation of band 3 lateral mobility is through interactions of band 3 cytoplasmic domain and the erythrocyte skeleton. The evidence comes from studies in which a dramatic increase in the lateral mobility of band 3 was observed in erythrocytes in which the skeleton had been either disrupted or depleted. (Golan and Veatch, 1980; Sheetz et al., 1980)

A potentially related and interesting observation is that marked membrane rigidity and immobilization of band 3 have now been described in Southeast Asian ovalocytes (Mohandas et al., 1984; Saul et al., 1984; Mohandas et al., 1992) and in llama elliptocytic red cells. A mutation in Southeast Asian ovalocytic band 3 has recently been characterized, and it results in the deletion of amino acids 400–408 at the boundary between the cytoplasmic and first transmembrane domains. (Tanner et al., 1991; Mohandas et al., 1992; Schofield et al., 1992) Secondary structure analysis of this mutant band 3 suggests that it deletes a flexible “hinge” region. Such a deletion might alter the conformation of the cytoplasmic domain resulting in an interaction with the skeletal network (Mohandas et al., 1992) (Fig. 5). To date llama band 3 has not been sequenced, and little is known about the biochemistry of the polypeptide. However, tantalizing data from cross-linking and proteolytic cleavage studies suggest structural differences between normal human and llama band 3 cytoplasmic domain (Khodadad and Weinstein, 1985). Thus, in Southeast Asian ovalocytic and llama red cell phenotypes, the primary structure of band 3 confers conforma-

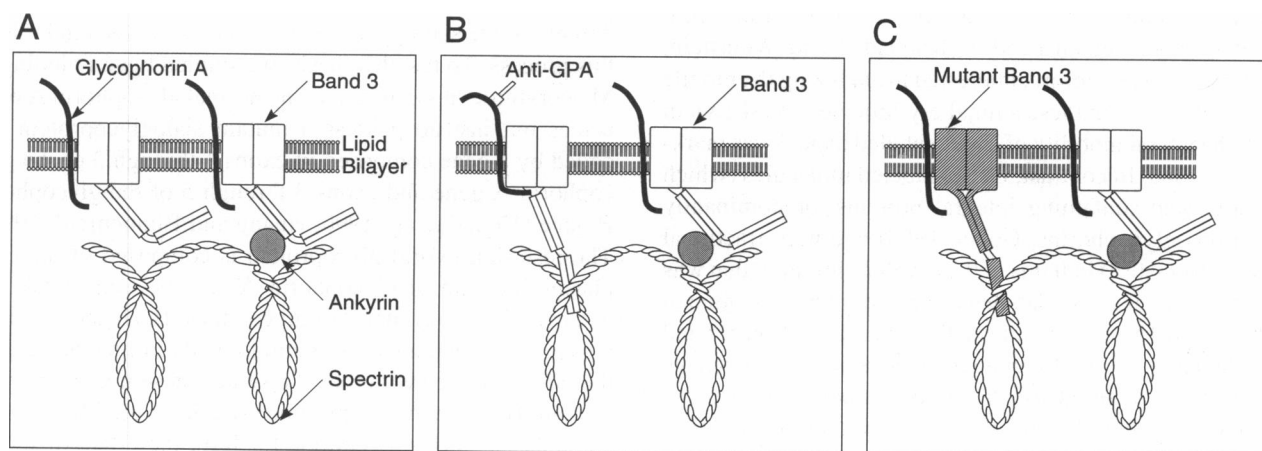


FIGURE 5 Model for molecular mechanism by which the cytoplasmic domain of band 3 induces membrane extensional rigidity. In (A) normal red cells the cytoplasmic domain of band 3 offers little resistance to the extension of spectrin tetramers during membrane deformation. In (B) glycophorin A-liganded cells and in (C) Southeast Asian ovalocytes antibody-induced and mutation-induced conformational changes in the cytoplasmic domain of band 3 results in entanglement in the spectrin network, sterically hindering the extension of spectrin tetramers.

tions of the cytoplasmic domains, which provide a different interaction with the skeletal network than that which occurs with normal human erythrocyte band 3. We speculate that these altered interactions between band 3 and the spectrin-based network result in membrane rigidification. We further speculate that a similar interaction between band 3 and the skeleton is perhaps induced by glycophorin A receptor ligands (Fig. 5). However, in the case of the Southeast Asian ovalocytic mutation, glycophorin A is mobile and band 3 immobile. In contrast, in normal erythrocytes glycophorin A and band 3 are mobile molecules and only become immobilized as a result of glycophorin A receptor ligand binding.

An alternative hypothesis that can account for the immobilization of glycophorin A and band 3 and the attendant increase in membrane rigidity is that ligand binding to glycophorin A induces a direct interaction between this protein and the membrane skeleton. This interaction in turn pulls the skeletal network close to the bilayer. This change in network topology prevents the spectrin network from undergoing the conformational changes necessary for membrane deformation. The observed immobilization of band 3 in this scenario could then result from steric effects on its diffusion as a result of the altered network topology. Although our data does not enable us to clearly distinguish between these two mechanisms, extensive recent data on the molecular basis for membrane rigidity of ovalocytic and llama red cells lead us, at the present time, to favor the model involving cooperative interaction between glycophorin A and band 3 in inducing membrane rigidity (Fig. 5).

Similar to glycophorin A, the glycoprotein members of the integrin family possess short cytoplasmic domains. In this widely expressed family of receptors the α - and β -subunits of the heterodimer have 50 amino acids or less in their cytoplasmic regions (Hynes, 1992). An important mode of signaling through integrins involves an association with the cytoskeleton. We suggest that the unexpected indication of cooperative action between glycophorin A and band 3 could represent a general mechanism for cytoskeletal linkage and transformation initiated by receptors with short cytoplasmic sequences.

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